IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
Lawrence E. Fosdick et al.
Application No. 10/685,125
Filed: October 13, 2003
Confirmation No. 4540
For: GLUCOSAMINE AND METHOD OF MAKING GLUCOSAMINE FROM MICROBIAL BIOMASS
Examiner: Elli Peselev
Art Unit: 1623
Attorney Reference No. 6682-65310-01

FILED VIA EFS
ON 4/30/08

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DECLARATION UNDER 37 CFR § 1.132

TZYY-JAN HAN, PH.D., being duly sworn does hereby declare and affirm the following:

That he received a Bachelor of Science degree in Agricultural Chemistry from National Taiwan University in 1977; that he received a Master degree in Chemistry from the University of Washington in 1981; that he received a degree of Doctor of Philosophy in Food Science from the University of Washington in 1987.

That he worked as a Postdoctoral Research Associate for the Horn Point Environmental Laboratory, University of Maryland, Cambridge, Maryland, from 1987 to 1992; that he worked as an Analytical Chemist for InterMountain Canola Company, Pennsauken, New Jersey, from 1992 to 1994; that he worked as an Analytical Chemist for Tones Spices Company, Ankeny, Iowa, from 1995 to 1997; and that he worked as the Quality Assurance Manager for Jeneil Biotech, Incorporated., Saukville, Wisconsin, from 1997 to 2003.

That he currently is a Senior Research Chemist and has been employed by Cargill, Incorporated since 2003. That his research specialties currently include analytical and process chemistry and that he actively participated in research further improving the developed process of glucosamine production from fungal biomass chitin and that he actively participated in the testing of conversion of chitin in fungal biomass to glucosamine as recited in Yang et al., Chinese Ag. Chem. Society, (1998) 36(6), 555-564 (working from the English translation of the same – attached hereto as Exhibit A).

I have reviewed the Yang reference and its method for determination of fungal mycelium in cereal. The Yang method includes two steps to convert chitin in fungal mycelium to glucosamine. I conducted tests to determine the amount of chitin that could be converted to glucosamine when fungal mycelium was directly digested under only the second step parameters of the Yang two-step method. In the second step of Yang the hydrolysate from the first step is diluted to 2 N HCl (7.3 wt%) and digested at 121°C for 2 hours.

The Test Materials and Methods were as follows:

1. Materials:

Fungal mycelium.

2. Methods:

a. Mycelium Dry Solids (DS): Mycelium DS was determined using a halogen balance.

b. Mycelium Digestion: Based on the DS, mycelium was mixed with concentrated HCl and water in a 300 mL pressurized glass bottle. The total volume of reaction mixture was about 200 mL; HCl concentration was 2 N (7.3 wt%), and the dry solids of mycelium were about 0.75 g/100 mL. The reaction mixture was heated at 121°C for 2 hours. At the end of digestion, the reaction mixture was immediately cooled in an ice bath.

c. Glucosamine and Glucose Determination: Glucosamine and glucose at Time 0 and 2 hour samples were determined by Dionex HPAE-PAD (high performance anion exchange-pulsed amperometric detection) chromatography using a Dionex CarboPac PA-20 column. Samples were diluted 200-fold before testing. External standards were used for quantitation.

Results

The average yield of conversion of chitin in the biomass to glucosamine was 25.8%. Specifically, the following test results were obtained:

wt.% of Dry Biomass

Digest 1	GlcN	Glc	**Yield%**
Time 0	0.00%	1.50%	
2 hr	5.77%	39.54%	**24.0**

wt.% of Dry Biomass

Digest 2	GlcN	Glc	
Time 0	0.00%	1.57%	
2 hr	6.60%	37.37%	**27.5**

Yang Process
Average Percent
Conversion 25.8

Only glucose was detected at Time 0
Glc = glucose
GlcN = Glucosamine
Yield based on 24 wt% chitin in dry biomass (see Specification, Table 3).

The undersigned declares that all statements made herein of his knowledge are true and that all statements made on information and belief are believed to be true and further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Executed at the place and date opposite the signature below.

Tzyy-Jan Han
Tzyy-Jan Han, Ph.D.

At Eddyville, IOWA
(City and State)
on this 28th day of April, 2008.

Introduction

Rice and corn are important cereals. The loss of these cereals caused by fungal contamination is more than 10%. (1) Besides the spoilage of cereals caused by the fungal contamination, some of the toxins generated by fungus are carcinogenic, which are harmful to the health of people and animals. Therefore it is very important to prevent the cereal grain from contamination, providing clean and safe food to people and animals. Quantification of mycelium in the grain is a method to evaluate the fungal contamination. Chitin, a polymer of (β-1, 4)-N-acetyl-D-glucosamine, is the major component in fungal mycelium cell wall. After acid hydrolysis, the resulting glucosamine can be quantified using colorimetric method to determine the amount of mycelium. (3) *Aspergillus*, *Penicilium* and *Fusarium* are common contamination fungi in corn and rice and also the major sources producing fungal toxin. In this article, we first discussed the acid hydrolysis of mycelium of *Aspergillus parasitisticus*, *Penicillium citrinum*, *Fusarium oxysporum*, the recovery after addition of mycelium to cereal and the effect of incubation conditions on glucosamine content in mycelium. Therefore the mycelium content can be accurately determined and presented as an evidence for cereal contamination.

Experimental Materials and Method

Experimental Materials

1. Strain
   Aspergillus parasiticus CCRC 30117, *Penicillium citrinum* CCRC 31620, *Fusarium oxysporum* CCRC 32121, purchased from Preservation Center of Cultures, Xinzhu Food Industry Development Institute
2. Chemicals and Materials
   (a) Medium
   Potato dextrose agar (PDA), brain heart infusion broth (BHI broth), potato dextrose broth (PD broth). Purchased from Difco(Detroit, MI, U. S. A.)
   (b) Reagent
   Potassium hydrogen sulfate, ferric chloride, sodium nitrite purchased from Nacalaitesque (Kyoto, Japan), ammonium sulfate, D(+)-glucosamine hydrochloride, 3-methyl-2-benzothiazolinone hydrazone (MBTH), chitin (food grade) purchased from Sigma (St. Louis, MO, U.S.A.).
   (c) Cereal
   Penglai rice used in the experiments was grown in Yuanlin and purchased from supermarkets. Machi corn purchased from grocery stores.

Experimental Methods

1.Preparation of spore suspension

EXHIBIT
A

The cultures were inoculated on PDA plate medium and grown at 30°C. After 4 days, the PDA was washed with sterile water containing Tween 80 and spore suspension was prepared with the spores scratched from PDA using inoculating loop. Adjustment of spore suspension concentration is based on Tuite method, After the suspension was mixed, a sample was taken for counting the spore numbers with hemacytometer and then the suspension was diluted with 0.1% Tween 80 to adjust the concentration to 10^6 spores/mL. The suspension was stored at 4°C in refrigerator.

2. Preparation of fungal mycelium powder

One million (10^6) spores were inoculated in 50 mL BHI broth (in a 250 mL Erlenmeyer flask) at 25°C at 120 rpm in a shaking incubator for several days. The mycelium in medium was filtered through Whatman No. 1 filter paper under water aspirator, vacuum pump and washed three times with 10 mL sterile distill water. The collected mycelium was freeze dried and grinded into powder with homogenizer. The powder was stored at –20°C in a refrigerator for future use.

3. Acid hydrolysis

According to the Desgranges's method, the sample (0.3-1.0g) was mixed with 10 mL of 10 N HCl solution and reacted at 20°C for 16 hours. Then the hydrolyzed sample was added to 40 mL distilled water and placed in an autoclave at 121°C for 2 hours, The pH of the sample was adjusted to 7.0 and diluted the total volume to 100 mL with distilled water. The colorimetric method was used for quantification.

4. Colorimetric method for quantification

According to Ride and Drysdale's method, to 1.5 mL diluted hydrolytic solution of fungal mycelium, 1.5 mL of 5% $KHSO_4$ and 1.5 mL of 5% $NaNO_2$ were added and shaked for 15 min. Then the solution was centrifuged at 5000 rpm for 2 minutes. 1.5 mL of the supernant was mixed with 0.5 mL 12.5% $NH_4SO_3NH_2$ followed by shaking for 5 minutes. To the boiled water, 0.5 mL of 0.5% MBTH was added and reacted for 3 minutes. After the solution is cooled down, the adsorption at 650 nm was measured. Standard curve is determined using glucosamine-HCl as standards. A series of standard solution 0, 10, 20, ...100 μg/mL solution were made up for colorimetric measurement.

5. Accuracy of chitin quantification using chemical method

   (a) Acid hydrolysis of chitin

   Three tenth to one gram (0.3-1.0 g) chitin standard was weighed and hydrolyzed in acid. (see above). The colorimetric method was used for the quantification of chitin. The degree of chitin hydrolysis is calculated as follow:

Degree of chitin hydrolysis (%)=$W_1/(W_0 \times 0.88) \times 100\%$

W_0: weight of chitin
W_1: amount of glucosamine measured after chitin hydrolysis
0.88: factor for theoretical glucosamine content after complete chitin hydrolysis

(b) Recovery of mycelium in cereal

Fifty and a hundred mg fungal mycelium were weighed and mixed with 1 g cereal powder (20 mesh) respectively. The controls were pure mycelium and pure cereal. After acid hydrolysis of the mixture, colorimetric method was used for the quantification.

Recovery of mycelium (%) = $(W_2-W_1)/W_0 \times 100\%$
W_0: amount of glucosamine in mycelium
W_1: amount of glucosamine in cereal
W_2: total amount of glucosamine in cereal with added mycelium

(c) Quantification of glucosamine before and after cereal acid hydrolysis

One g cereal powder was hydrolyzed in acid followed by quantification with colorimetric method. The amount of glucaosamine in 1 g cereal without acidic hydrolysis was also measured with colorimetric method.

6. Factors for affecting glucosamine content in mycelium

(a) Incubation time

Based on the conditions of mycelium incubation described above, the mycelium powder was prepared by incubating the spore suspension for 2, 3, 5,7 days. After acidic hydrolysis of mycelium, glucosamine in mycelium was measured with colorimetric method.

(b) Incubation temperature

Based on the conditions of mycelium incubation described above, the mycelium powder was prepared by incubating the spore suspension at 25 and 30°C for 2, 3, 5,7 days respectively. After acidic hydrolysis of mycelium, glucosamine was measured with colorimetric.

(c) Medium

Based on the conditions of mycelium incubation described above, the mycelium powder was prepared by incubating the spore suspension in BHI and PD broth at 25°C at 120rpm for 5 and 7 days respectively. After acidic hydrolysis of mycelium, glucosamine was measured with colorimetric method.

(d) Shaking incubation and still incubation

Based on the conditions of mycelium incubation described above, the mycelium powder was prepared by incubating the spore suspension at 25°C at 120rpm for 5 and 7 days respectively. The same spore suspension was inoculated and incubated at the same temperature without shaking. After acidic hydrolysis of mycelium, glucosamine was measured with colorimetric method.

7. Measurement of dry weight of mycelium

The obtained medium from experiments above was filtered through preweighed Whatman No. 1 filter paper under vacuum to separate mycelium. The collected mycelium was dried at 150°C in an oven till no changes in weight. The dry mycelium weight was calculated by subtracting filter paper weight from the constant weight of mycelium and filter paper.

8. Statistical analysis

The Statistical analysis system: SAS 2.0 was used in analyzed the experimental results.

Results and Discussions

Measurement of Glucosamine

1.Acidic hydrolysis of Chitin

Chitin in organism was boned with O-glycosidic linkage in parallel or anti-parallel orientation. Due to strong H bond, chitin is chemically and physically stable. Therefore the chitin was hydrolyzed to small molecules such as N-acetyl glucosamine or glucosamine for quantification. The degree of hydrolysis greatly affects the accuracy of chemical quantification method. In the experiments described in the article, the deacetylation of chitin was proceeded in concentrated HCl at 20°C for 16 hours and then the depolymerization of chitin was undergone in 2N HCl at 121°C for 2 hours to generate glucosamine. Table 1 gave the hydrolysis degrees of 0.3-1.0 g chitin standards after acidic treatment. The results showed the acidic hydrolysis degrees of chitin using the method described above were more than 90%, the average was 94.5%, which was almost one time higher than degree of salt hydrolysis 50%. It proves that the conversion of acid hydrolysis is higher than the salt hydrolysis. The reason is that salt hydrolysis acts only on acetyl bond but acid hydrolysis can also depolymerize the chitin to release more glucosamine.

3. The recovery of fungi mycelium added in rice and corn

The accuracy in evaluation of fungal contamination in cereal is not only dependent on the hydrolysis degree of chitin in fungi mycelium, but also determined by recovery of fungal mycelium added in cereal. Table 2 is the recovery of fungal mycelium added in rice and corn. The results showed after the addition of 50-100 mg mycelium in rice and corn, the recovery of mycelium from the same culture is similar except *P. citrinun* recovery in rice. The difference in recovery may be caused by glucosamine baseline in cereal sample. Additionally Table 2 also exhibited there was no significant difference between recovery of mycelium added in corn and the recovery of mycelium added in rice. To rice and corn, *A. parasiticus, P. citrinum and F. oxysporum* was added, the average recovery of the mycelium is 69%, 82% and 73% respectively. The average of recovery in all is 75%, which are higher than 61% recovery of Arima and Uozomi method (10), using concentrated sulfuric acid hydrolysis and 69% recovery of Swift method, using 6 N HCl hydrolysis.(11)

2. Factors affecting the level of glucosamine in the mycelium

1). Incubation time

Cousin et al (12) reported that the level of glucosamine in the mycelium cell wall would increase when incubation time increased. Table 3 illustrates the changes of the amount of mycelium and glucosamine after various incubation times in three different test strains. In terms of the glucosamine content, *A. parasiticus* incubated in BHI medium at 25 °C with shaking at 120 rpm for 2 days, the glucosamine content increased from 132 mg/g mycelium on the second day to 198 mg/g mycelium on the fifth day. But the glucosamine level dropped slightly to 181 mg/g mycelium on the 7th day. However, the glucosamine content in the mycelium of *P. citrinum* increased continuously from 122 mg/g on the 2nd day to 229 mg/g on the 7th day. *F. oxysporum* also increased continuously from 101 mg/g on the 2nd day to 130 mg/g on the 7th day. This was due to the precipitation of chitin in mature mycelia when fungal growth reached stationary phase. Therefore, the content of glucosamine in the mycelium could increase continuously. But in the *A. parasiticus* mycelium, the glucosamine content reached maximal level on the 5th day. The glucosamine content dropped 8.8% on the 7th day from the maximal level on the 5th day. This was due to simultaneous biosynthesis and breakdown of glucosamine in the mycelium cell wall during growth period. There was subtle dynamic equilibrium occurred in the cell wall (14). Therefore in the early stage of fungal growth, the changes of glucosamine content in the mycelium reflected the rapid growth of mycelia (15). When the carbon source in the media depleted or when cells are under starvation, fungus will recycle chitin in the cell walls by breaking down the old cells (16). Although glucosamine is one of the components of the cell wall, it can be utilized through metabolic pathway (17). In other words, old cells were broken down to facilitate the growth of new cells and this results in the decrease of the level of glucosamine in the mycelium. Herrera (18) reported that the glucosamine content in *Aspergillus* cell wall ranging from 13.8 – 17.1% of the dry weight of mycelium. This agrees with our finding of glucosamine in the mycelium of *A. parasiticus* that is 13.2 – 19.8%. The glucosamine content in *P. citrinum* dry mycelium ranged from 12.2 – 22.9% that agrees with the glucosamine content in dry mycelium of *Penicillium* genus. The *F. oxysporum* mycelia contain 102 – 130 mg glucosamine per gram that is similar to the results reported by Cochran and Vercellotti(20).

The dry weight of mycelium is often used as an index of fungal growth (2). When the cultivation time increases, the amount of mycelia growth also increases. *A. parasiticus, F. oxysporum,* and *P. citrinum* reached their maximal growth after 5, 3, and 7 days, respectively. Our results showed that the amount of mycelia started to decrease after fungal growth reached maximal level. This observation was also reported in other literature(20). This is due to the inhibitory effect of glucosamine on the metabolism of monosaccharide, such as glucose and mannose in the cell wall. Therefore, glucosamine could inhibit the growth of fungal mycelia(21). Due to the accumulation of glucosamine in fungal mycelia, when the time of incubation increased, the growth of mycelia was inhibited(22).

2). Incubation temperature

Most fungus can grow at 0 – 54 °C. But the ideal growth temperature is 21 – 38 °C. Literature reported that high incubation temperature could enhance the hydrolytic enzyme activities in the cell wall, therefore, inhibited the biosynthesis of chitin, which resulted in the self-lysis of mycelium(23). In this study, room temperature 25 °C and the average Taiwan Summer temperature 30 °C were used as experiment temperatures. Table 3 shows that as the incubation time increased, both the growth of mycelium, and the glucosamine content also increased gradually. Regarding glucosamine content, its level rapidly increased from 5 to 7 days at 30 °C. To *A. parasiticus,* the amount of glucosamine in the mycelium was higher in the first 5 days at 25 °C than that at 30 °C incubated for the same length of time. *P. citrinum* incubated at 25 °C had glucosamine content higher than that incubated at 30 °C during the whole period of time. In contrary, when *F. oxysporum* incubated at 30 °C, the glucosamine content in the mycelium increased slightly. In comparison of the maximal glucosamine content in each fungus, except for *P. citrinum, A. parasiticus,* and *F. oxysporum* both had higher glucosamine at 30 °C than 25 °C.. This is because chitin accumulation rate was faster at higher temperature than that at lower temperature(24).

In studying the effect of incubation temperature on the mycelium growth, the results indicated that the growth rate of *A. parasiticus* and *F. oxysporum* were faster than that of *P. citrinum* at 30 °C. Based on the maximal level of glucosamine content, at 30 °C, BHI media with 120 rpm, *F. oxysporum* grew better than either , *A. parasiticus,* or *P. citrinum.* The same pattern was observed at 25 °C as well. In terms of growth rate, *A. parasiticus,* and *P. citrinum* grew faster at 30 °C than 25°C. The incubation temperature did not affect the growth rate of F. oxysporum.

3). Medium

The nutrients in the medium, such as carbon source, nitrogen source, carbon/nitrogen ratio have great effect on fungal growth and the accumulation of chitin(25). Moulki and Bonaly(26) reported that certain ingredients in the medium would cause greater amount of glucosamine accumulated in the fungal cell wall. But it was not the main factor. Medium containing brain-heart infusion (BHI) broth is often used to isolate fungus in clinical medicine. It is good culture ingredient for microorganism has strict nutrition requirements. However, potato dextrose broth is common medium for yeast and fungus(27). Therefore, these two medium were used in this study to investigate how mycelium growth and glucosamine content were affected by different type of medium. The changes of glucosamine in the mycelium and mycelium growth of all three fungi incubated at 25 °C in these two medium from 5 to 7 days were listed in Table 4. The mycelium growth and glucosamine content in all three fungi incubated in brain-heart infusion medium were higher than those incubated in potato dextrose broth medium.

4). Shaking and stationary incubation

Table 5 has the results of the study of the effect of shaking or stationary incubation on the glucosamine content in the mycelium and the growth of mycelium. Under the same number of days of incubation, except for *F. oxysporum* which had higher level of glucosamine in the mycelium when incubated in stationary state for 5 days. The rest two fungi had higher amount of glucosamine when incubated with shaking. The results indicated that when oxygen level reduced during incubation, the glucosamine content in certain fugal mycelia decreased also. In terms of the effect on mycelium growth, Table 5 showed that within 5 days, the mycelium growth under shaking was greater than that under stationary incubation. However, at the 7th day, except for *P. citrinum,* the other two fungi had greater mycelium growth under stationary incubation than incubation with shaking.

CONCLUSION

The original goal of this study was to predict the amount of mycelium in grains by measuring the glucosamine content in rice or corn. So that we could use this method to understand how grains were contaminated by fungus during harvest and storage. Consequantly, chitin in the mycelium (0.3 – 1.0 g) was found hydrolyzed by hydrochloric acid. The average rate of hydrolysis was 94.5%. When 50 – 100 mg of mycelium of *A. parasiticus, P. citrinum, and F. oxysporum* were added to rice or corn, the recovery rate of mycelium was calculated by the measurement of glucosamine content. It was found that the recovery rate of certain mycelium depending on the species of the microorganism (e.g. *P. citrinum*). The overall average recovery of the above mentioned three strands of fungal mycelia were about 75%. This study found that the glucosamine content in the mycelium was not just affected by the microorganism itself, but also affected by the type of medium, incubation method, incubation temperature, and time. Therefore, when acidic hydrolysis method was used to quantify the amount of mycelium in grains, the above mentioned several factors need to be considered. So the fungal contamination in grains could be estimated more accurately.

ACKNOWLEGEMENT

This research project was sponsored by the National Science Council financial support (NSC85-2321-B002-049).

LITERATURES